Effect of dextran sulphates on thrombin activity

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SUMMARY To clarify the action of dextran sulphate, a heparin analogue, in the clotting of fibrinogen by thrombin, determinations were carried out on the clotting activity, the release of fibrinopeptides from fibrinogen, and the hydrolytic activity of thrombin against a peptide chromogenic substrate in the absence or presence of antithrombin III (heparin cofactor). It was shown that dextran sulphate itself inhibited thrombin activity, and its inhibition was dependent on the molecular weight and the sulphur content of the dextran sulphate. Although heparin markedly enhanced the antithrombin activity of antithrombin III, dextran sulphate did not activate antithrombin III.

Heparin is composed of amino sugar and uronic acid residues, and its molecular weight is roughly 10000-20000 (Jeanloz, 1975). As its synthesis has not yet been completely successful, a structural analogue of heparin (heparinoid), dextran sulphate, which is a synthetic dextrose polymer, has been investigated for its various biological and pharmacological actions. So far several influences of dextran sulphate on coagulation and the fibrinolytic system, along with its lipolytic, anticholesterolaemic, and anti-hyaluronidase activities, have been reported (Douglas, 1956; Yamada and Kuzuya, 1962). Of these actions, the anticoagulant activity is the most significant, but its mechanism seems to be very complicated and remains unclear.

The present study was designed to clarify the anticoagulant mechanism of dextran sulphate on the fibrinogen-fibrin conversion process, fibrinopeptide release from fibrinogen by thrombin, and the hydrolytic activity of thrombin against a peptide chromogenic substrate in the absence or presence of antithrombin III. The relationships between molecular weight or sulphur content and the anticoagulant activity of dextran sulphate were also investigated.

Material and methods

A 0.1M NaCl-0.05M Tris-HCl buffer (pH 7.5) was used throughout.

Fibrinogen (97-98% clottability) was purified from human plasma by the method of Blombäck and Blombäck, (1956). Partially purified bovine thrombin was prepared from thrombin-Mochida (Mochida Pharm., Tokyo, Japan) by chromatography on

DEAE-cellulose (Whatman DE-32, W.R. Balston, England) according to the method of Yin and Wessles (1968). The specific activity of thrombin prepared by this method was 1900 NIH units per mg protein. It was made up as a stock solution in a concentration of 54 μ g (100U) per ml in the Tris-HCl buffer and stored in small aliquots in plastic tubes at -70°C .

Antithrombin III (heparin cofactor) was prepared from human plasma by the method of Machovich *et al.* (1975) using barium sulphate adsorption, defibrination by heat, adsorption by aluminium hydroxide, fractionation with ammonium sulphate, gel filtration on Sephadex G-200 (Pharmacia Chem., Uppsala, Sweden), and chromatography of DEAE-cellulose (Serva, Seikagaku Kogyo, Tokyo, Japan). The antithrombin III prepared by this method was concentrated 300-fold over plasma and showed essentially a single band on SDS-gel electrophoresis. This antithrombin III was dialysed against the Tris-HCl buffer and then stored at -70°C until used.

Quantitative determination of antithrombin III was carried out with immunodiffusion plates (M-Partigen, Behring Inst, West Germany) (Fahey and Mckelvey, 1965; Mancini *et al.*, 1965).

Solutions of sodium dextran sulphate (Kowa Pharm., Tokyo, Japan) of molecular weight (MW) 3500 (sulphur content; S% = 5.3 or 18.0), MW 7500 (S% = 5.8 or 18.1), MW 10 000 (S% = 17.9), MW 50 000 (S% = 19.3), and MW 200 000 (S% = 19.0) were made at the specified concentrations in the Tris-HCl buffer and stored at 4°C until used.

Sodium heparin (165 units/mg, Batch TCP 0730) was obtained from Wako Pure Chem, Tokyo, Japan and Phenanthrenquinone reagent was purchased from Nakarai Chem., Kyoto, Japan.

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Peptide chromogenic substrate, S-2238 (H-D-Phe-Pip-Arg-pNA, 2HCl), was purchased from KABI Diagnostica, Stockholm, Sweden.

THROMBIN CLOTTING TIME

Clotting time was measured with a BBL-Fibrometer (BBL-Comp) according to the method of Hashimoto *et al.* (1975).

0.1 ml of a dextran sulphate solution or heparin of various concentrations (from 4×10^{-5} to 40 mg/ml), 0.1 ml of thrombin (10.5 μ g/ml), and 0.1 ml of the buffer or antithrombin III (200 μ g/ml) were preincubated at 37°C for 2 minutes, and then 0.1 ml of fibrinogen solution (2.64 mg/ml) was added to determine the clotting time.

DETERMINATION OF FIBRINOPEPTIDES RELEASED FROM FIBRINOGEN

Antithrombin activity was determined by measuring the inhibitory effect by thrombin on the release of fibrinopeptides from fibrinogen. The release of fibrinopeptides from fibrinogen was quantified by Yamada and Itano's modification of the phenanthrenquinone reaction (Yamada and Itano, 1966), the specific assay for detecting an arginine or a peptide containing arginine residues: 1 mol fibrinopeptide A or B contains 1 mol arginine residue, and 1 mol fibrinogen releases 2 mol each of fibrinopeptides A and B; thus, 4 mol fibrinopeptides are released from 1 mol fibrinogen cleaved by thrombin.

The assay procedure was carried out as follows: 250 μ l fibrinogen (2.64 mg/ml, 2 nmol fibrinogen in the final reaction mixture), 250 μ l heparin, dextran sulphate (2×10^{-2} mg/ml) or (for the control) buffer, and 50 μ l thrombin (1.05 μ g/ml) were mixed and incubated at 37°C for 0, 5, 10, 15, 20, 30, 45, and 60 minutes. At specified incubation times the reaction was stopped by the addition of 50 μ l chilled 100% (w/v) trichloroacetic acid.

After removal of the denatured fibrin clot by centrifugation at 2000 *g* for 10 minutes, the supernatant was washed three times with the same volume of chilled petroleum ether and ethyl ether mixture (1:1) and then with the same volume of ethyl ether three times. To 400 μ l of the water layer, 1.5 ml of 0.1 mM phenanthrenquinone reagent (dissolved in 100% ethanol) and 0.2 ml of 0.2N NaOH were added. After 30 minutes 2.5 ml of 1N HCl was added, and the fluorescence of the reaction mixture was determined with an excitation wavelength of 365 nm and an emission wavelength of 395 nm by fluorophotometer (MPF-2 type, Hitachi Seisakusho, Japan).

To determine the effect of dextran sulphate or heparin on the release of fibrinopeptides from fibrinogen in the presence of antithrombin III, the following experiments were carried out: 100 μ l

dextran sulphate or heparin at a concentration of 5×10^{-3} and 5×10^{-2} mg/ml, 50 μ l thrombin (1.05 μ g/ml), and 100 μ l antithrombin III (10 μ g/ml) or buffer (for the control) were preincubated at 37°C for 2 minutes, and then 250 μ l fibrinogen solution (2.64 mg/ml) was added. The mixture was incubated at 37°C for 30 minutes. The reaction was stopped by the addition of 50 μ l chilled 100% (w/v) trichloroacetic acid.

DETERMINATION OF HYDROLYTIC ACTIVITY OF THROMBIN AGAINST PEPTIDE CHROMOGENIC SUBSTRATE

The hydrolytic activity of thrombin with dextran sulphate in the absence or presence of antithrombin III was measured by the spectrophotometric technique with a peptide chromogenic substrate, H-D-Phe-Pip-Arg-pNA. The reaction was carried out as follows using 0.1M NaCl-0.05M Tris-HCl buffer (pH 8.4) throughout: 100 μ l of various concentrations of dextran sulphate or heparin (from 5×10^{-5} to 5 mg/ml) and 50 μ l of antithrombin III (50 μ g/ml) or buffer were mixed and incubated at 37°C for 2 minutes in the warmed cell, and then 50 μ l thrombin (92.6 μ g/ml) was added. After 30 seconds exactly, 300 μ l prewarmed substrate solution (0.5 mM) was added to the incubation mixture and continuous recording at 405 nm was started immediately. Δ OD at 405 nm was calculated after a recording period.

Results

EFFECTS OF DEXTRAN SULPHATE

On thrombin clotting time

Figure 1 shows the effect of dextran sulphate or heparin on the thrombin clotting time in the absence of antithrombin III.

Although heparin did not prolong the clotting time, even at high concentrations, dextran sulphate alone did. The greater the molecular weight or sulphur content of the dextran sulphate, the greater the prolongation of the clotting time. For some unknown reason the inhibitory effect was not found in the dextran sulphate, the molecular weight of which was 7500 ($S\% = 5.8$).

As shown in Fig. 2, small amounts of heparin prolonged the clotting time more in the presence of antithrombin III than in its absence. Dextran sulphate also apparently prolonged the clotting time in the presence of antithrombin III, and this prolongation seemed to be caused by the higher molecular weight dextran sulphate than by that of low molecular weight.

On release of fibrinopeptides from fibrinogen

Figure 3 shows the effect of dextran sulphate on the

Fig. 1 Effects of dextran sulphates or heparin on thrombin clotting time in the absence of antithrombin III. The dextran sulphate samples have the following properties: (1) MW 3500 ($S\% = 5.3$), (2) MW 3500 ($S\% = 18.0$), (3) MW 7500 ($S\% = 5.8$), (4) MW 7500 ($S\% = 18.1$), (5) MW 10 000 ($S\% = 17.9$), (6) MW 50 000 ($S\% = 19.3$), (7) MW 200 000 ($S\% = 19.0$), (8) heparin.

Fig. 2 Effects of dextran sulphates or heparin on thrombin clotting time in the presence of antithrombin III. Samples as in Fig. 1.

Fig. 3 Effects of dextran sulphate or heparin on the release of fibrinopeptides from fibrinogen in the absence of antithrombin III. Ordinate shows moles fibrinopeptides released from 1 mol fibrinogen. The dextran sulphate samples have the following properties: (1) Control (buffer), (2) MW 3500 ($S\% = 5.3$), (3) MW 3500 ($S\% = 18.1$), (4) MW 7500 ($S\% = 5.8$), (5) MW 7500 ($S\% = 18.1$), (6) MW 10 000 ($S\% = 17.9$), (7) MW 50 000 ($S\% = 19.3$), (8) MW 200 000 ($S\% = 19.0$), (9) heparin. Experimental conditions are detailed in material and methods.

release of fibrinopeptides from fibrinogen in the absence of antithrombin III. In controls that did not contain dextran sulphate fibrinopeptides were gradually released during incubation until 30 minutes later about 4 mol peptides had been released from 1 mol fibrinogen. Heparin alone did not affect the release of fibrinopeptides. In the presence of sodium-DS, the release of peptides was considerably altered. The dextran sulphate of MW 3500 ($S\% = 5.3$) and MW 7500 ($S\% = 5.8$) did not affect the release of peptides at a final concentration of about 10 $\mu\text{g/ml}$. The dextran sulphate with high sulphur content (17.9-19.3%) exerted a suppressive influence on the rate of release and the amount of released fibrinopeptides. In high sulphur content dextran sulphates the inhibition of fibrinopeptide release was found to be proportional to the molecular weight of the dextran sulphate. These observations demonstrated the antithrombin activity of dextran sulphate and the dependence of this activity on the intra-molecular sulphur content and the molecular weight of the dextran sulphate.

Table 1 shows the effects of various concentrations of dextran sulphate or heparin on the release of fibrinopeptides 30 minutes after the addition of

Table 1 Effects of dextran sulphate or heparin on release of fibrinopeptides from fibrinogen in the absence or presence of antithrombin III

Heparinoids	Released fibrinopeptides (mol) from 1 mol fibrinogen			
	Concentration of heparinoids			
	1 µg/ml		10 µg/ml	
	Antithrombin III		Antithrombin III	
	-	+	-	+
Dextran sulphate				
MW 3 500 (S% = 5.3)	4.1	3.2	3.9	3.0
MW 3 500 (S% = 18.0)	3.7	2.8	3.1	2.0
MW 7 500 (S% = 5.8)	3.9	3.0	3.8	2.9
MW 7 500 (S% = 18.1)	3.0	1.9	2.2	1.1
MW 10 000 (S% = 17.9)	2.6	1.7	1.6	0.6
MW 50 000 (S% = 19.3)	2.2	1.1	1.2	0.1
MW 200 000 (S% = 19.0)	2.0	0.8	1.1	0
Heparin	4.2	0	4.0	0
Buffer (control)	4.1	3.1	3.8	2.7

Fig. 4 Effects of various concentrations of dextran sulphates and heparin on hydrolytic activity of thrombin. Thrombin activity with dextran sulphate or heparin was measured by the release of pNA from H-D-Phe-Pip-Arg-pNA per min and per IU of thrombin at 37°C.

Symbols for dextran sulphate or heparin as follows: —●— MW 3500 (S% = 5.3); —○— MW 3500 (S% = 18.0); —□— MW 7500 (S% = 5.8); —■— MW 7500 (S% = 18.1); —▲— MW 10000 (S% = 17.9); —△— MW 50000 (S% = 19.3); —▼— MW 200000 (S% = 19.0); —×— heparin.

thrombin to the fibrinogen solution in the absence or presence of antithrombin III. At a concentration of 1 µg/ml heparinoids, and in the absence of antithrombin III, high molecular weight dextran sulphate had a moderately inhibitory effect on the release of fibrinopeptides. In the presence of antithrombin III, although heparin enhanced the inhibition of peptide release, dextran sulphate did not so enhance the peptide release. The amount of peptides released from fibrinogen in the presence of dextran sulphate

and antithrombin III was nearly equivalent to the total amount of peptides released from fibrinogen in the presence of dextran sulphate alone and of antithrombin III alone. These phenomena were observed when the concentration of heparinoids increased 10 fold. Therefore, it is suggested that antithrombin III does not mediate the antithrombin activity of dextran sulphate.

Figure 4 shows how dextran sulphate and heparin affect the hydrolytic activity of thrombin acting on a peptide chromogenic substrate, H-D-Phe-Pip-Arg-pNA, in the absence of antithrombin III. Dextran sulphates with a high sulphur content and high molecular weight directly inhibited the hydrolytic activity of thrombin. However, low sulphur content dextran sulphates and heparin did not show this inhibitory effect. Moreover, in the presence of antithrombin III (Fig. 5), the inhibition of the hydrolytic activity of thrombin was enhanced only by heparin and not by dextran sulphates. The inhibitory effect of a dextran sulphate on hydrolysis in the presence of antithrombin III was nearly equivalent to the total effect of dextran sulphate alone and of antithrombin III alone.

Discussion

This study showed the influence of dextran sulphates, a structural analogue of heparin, with different molecular weights and/or sulphur contents, on thrombin activity in the absence or presence of antithrombin III (heparin cofactor).

Many investigators have found that heparin alone does not affect thrombin directly but rather acts through antithrombin III (Abildgaard, 1969; Rosenberg and Damus, 1973; Bleich and Rosenberg, 1975). Supposedly, heparin acts merely as a catalyst

Fig. 5 Effects of various concentrations of dextran sulphates and heparin on hydrolytic activity of thrombin in the presence of antithrombin III. Thrombin activity with dextran sulphate or heparin in the presence of antithrombin III (2.5 μ g/500 μ l) was measured by the release of pNA from H-D-Phe-Pip-Arg-pNA per min and per IU of thrombin at 37°C. For explanation of symbols see Fig. 4.

for the otherwise sluggish formation of a 1:1 molar complex between antithrombin III and thrombin.

We confirm the action of heparin on thrombin and antithrombin III as described in previous papers (Rosenberg and Damus, 1973; Bleich, 1975). However, the present study demonstrates that dextran sulphate alone had an inhibitory activity on thrombin. The thrombin inhibitory activity of dextran sulphate was indicated not only by the thrombin clotting time and the determination of fibrinopeptides released from fibrinogen, but also by the determination of the hydrolytic activity of thrombin against a peptide chromogenic substrate. The thrombin inhibitory activity was dependent upon the concentration and also upon the molecular weight of the dextran sulphate. For the same molecular weight of dextran sulphate, the higher the intramolecular sulphur content, the stronger the thrombin inhibitory activity.

Muzaffar *et al.* (1972) reported that dextran alone binds and precipitates fibrinogen, and it also shortens the thrombin clotting time. Considering their results, we think that there are two possibilities for the inhibitory effect of dextran sulphate on fibrinogen clotting: (1) negatively charged sulphur groups of dextran sulphate bind to the basic amino acid residues, for example, arginine or lysine, of fibrinogen to produce steric hindrance against thrombin, or

(2) dextran sulphate binds directly to the thrombin and inhibits its action on fibrinogen.

The present experiment shows that dextran sulphate inhibited the amidolytic activity of thrombin against a peptide chromogenic substrate, and in other experiments we have found that dextran sulphate alone inhibited the esterolytic activity of thrombin against a synthetic substrate *p*-tosyl-L-arginine methyl ester (TAME) (Suzuki). Machovich *et al.* reported that heparin binds to thrombin and induces a conformational change, facilitating a complex formation between thrombin and antithrombin III (Machovich, 1975a; Machovich, 1975b; Machovich *et al.*, 1975). Recently, Smith and Craft (1976) reported that heparin alone forms a strong complex with thrombin in a 1:1 molar fashion. More recently, Nordenman and Björk (1978) found that heparin binds with a high affinity to thrombin, active as well as inactive, but not to prothrombin, and the binding of heparin to thrombin has no effect on the amidase activity. Therefore, we consider that dextran sulphate binds directly to the thrombin rather than to the fibrinogen and inhibits thrombin action on fibrinogen. However, there remains the alternative hypothesis that dextran sulphate binds to fibrinogen, thereby blocking thrombin action.

Moreover, to find whether dextran sulphates activate antithrombin III or not, we determined the release of fibrinopeptides from fibrinogen and the hydrolysis of a peptide chromogenic substrate by thrombin in the presence of antithrombin III. However, we did not find that dextran sulphates enhanced the antithrombin activity of antithrombin III.

Duncun and Chris reported that heparin and dextran sulphate (Pharmacia, MW 500 000) show similar binding characteristics for plasma pro-coagulants, but only heparin activates antithrombin III (Pepper and Prowse, 1977).

In conclusion, it is clear that dextran sulphates with a high molecular weight and a high sulphur content inhibit thrombin activity directly, but dextran sulphates do not activate antithrombin III. These characteristics of dextran sulphates differ from those of heparin.

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